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Heparanase Mechanisms in Melanoma Brain Metastasis

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14. ABSTRACT Heparanase (HPSE) is the dominant endoglycosidase (endo- $\beta$ -D-glucuronidase) in mammals and is an important tumorigenic, angiogenic, and pro-metastatic enzyme. Highest levels of HPSE activity have been consistently detected in cells with highest propensities to colonize the brain. This emphasizes the potential for therapeutically targeting this enzyme in brain metastasis in general, brain-metastatic melanoma (BMM) in particular. Of interest, SST0001 is a small-molecule, non-anticoagulant heparin with potent anti-HPSE activities. Objective of the work was to assess the abilities of SST0001 to interfere with HPSE-mediated cell signaling and actions, and ultimately affecting the modulation of BMM. Accordingly, this, by employing the pINDUCER lentiviral system, and performing heparanase gain-/loss-of-function investigations in melanoma cell clones highly metastatic to brain (high BMM cell lines); and translated findings in xenografts. This was done in the presence or absence of SST0001 treatment, employing the pINDUCER lentiviral system. We found that HPSE plays important roles in mechanisms modulating BMM onset. A new molecular mechanism was also identified by which HPSE mediates an alternative survival pathway in BMM cells, being modulated by SST0001 <i>in vitro</i> and <i>in vivo</i> . These investigations can contribute to the development of novel therapeutic strategies for BMM to improve patient outcomes.					
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## 1. INTRODUCTION:

Patients with brain metastatic melanoma (BMM) have, even with the best available treatments, a median survival of less than six months. Unfortunately, mechanisms underlying BMM remain largely unknown. The current study was designed to provide novel mechanisms underlying BMM disease. Its rationale pertains to findings by our laboratory as well as others showing that heparanase (HPSE) acts as a potent pro-tumorigenic, pro-angiogenic, and pro-metastatic enzyme (1-7). Heparanase is the only endoglycosidase in mammals cleaving heparan sulfate (HS) into fragments which retain biological activity. An established role for heparanase is to release growth and angiogenic factors avidly bound to extracellular matrix HS, regulating their levels and their binding to the respective tumor cell surface receptors, thus altering factors' biological potency. These functions are mediated by enzymatically active heparanase. Our work has implicated heparanase as a promoter of brain metastasis since the enzyme is most active in cells selected *in vivo* to possess highest capabilities to metastasize to brain, independently from the cancer type. We have demonstrated that heparanase is expressed in human BMM tissues and produced by glial cells (astrocytes) in response to a tumor-induced insult (1). This can foster metastatic growth within the brain microenvironment which is acidic, thus optimal for HPSE enzymatic action (1-4). Furthermore, recent discoveries have put forward the notion that heparanase possesses functions which are independent of its enzymatic activity, e.g., altering cell adhesion, signal transduction, cytoskeletal dynamics, and mediated by the unprocessed, latent form of the molecule (2, 3).

2. **KEYWORDS:** Brain-metastatic melanoma (BMM), Heparanase (HPSE), SST0001, pINDUCER.

## 3. OVERALL PROJECT SUMMARY:

This report represents progress made for the second year of CDMRP Discovery Award whose objective was to assess the relevance of regulating HPSE which result in consequent modulation of BMM onset. To this end, we employed the HPSE inhibitor SST0001. SST0001 is a prototypic non-anticoagulant

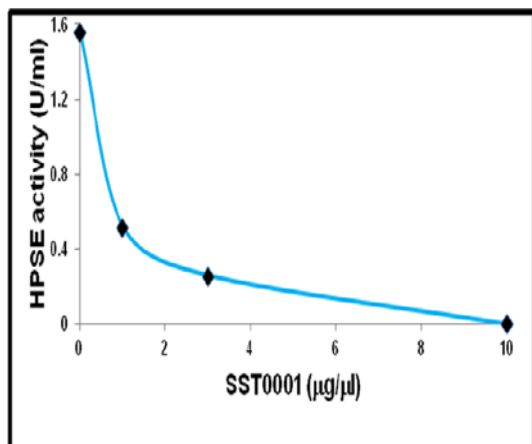


Figure 1. Dose-dependent inhibition of heparanase (HPSE) activity by SST0001. Aliquots of purified recombinant human HPSE were treated with SST0001 (0 - 10 µg/µl) for 2 hours at 37°C. HPSE activity (U/ml) was then determined using the Heparan Sulfate Degrading Enzyme Assay™ kit (cat. # MK412 Takara, Inc.) following manufacturer's protocol.

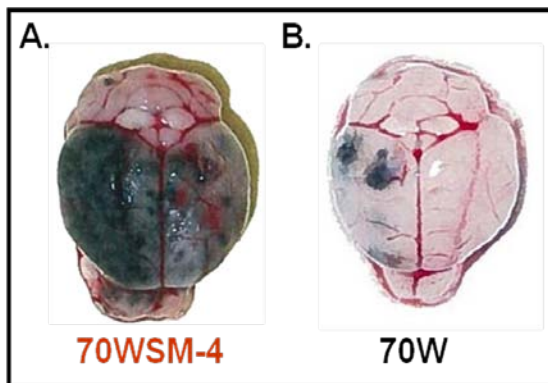
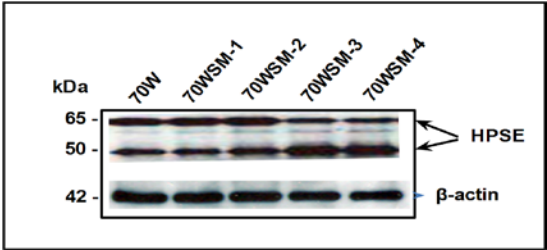


Figure 2. Brain metastasis formation by the human super BMM 70WSM-4 clone (A.) derived from brain metastatic 70W melanoma cells (B.). 70W cells were injected into the internal carotid artery of nude mice, BMM was assessed, tumors dissected and cultured *in vitro*. Cell cultures were then re-injected into mice and the recovery of BMM was repeated (four cycles of *in vivo* selection) obtaining the super BMM 70WSM-4 clone. Images of BMM from animals injected with an equal number of 70WSM-4 or 70W cells, and sacrificed at the same time are shown.

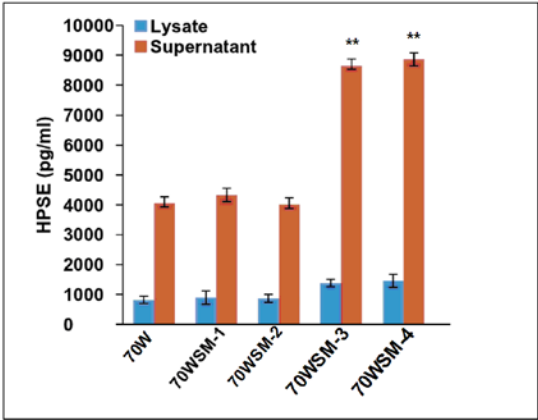
heparinoid and has emerged as a potent and specific inhibitor of purified HPSE (**Figure 1**). Further, it has been successfully used in models of multiple myeloma (7). Objective was to evaluate the efficacy of SST0001 in a panel of BMM cell lines with augmented propensities for BMM onset, eg, highly brain metastatic clones obtained by the sequential in vivo selection of 70W cells (70WSM1-4 clones), with clone 70WSM-4 being the most aggressive towards brain colonization (**Figure 2**). Sub-tasks have been as follows:

*Implement the use of pINDUCER lentiviral tool kit by cloning shRNA to HPSE. Transduce human brain-metastatic melanoma (BMM) cells with these lentiviral vectors.*

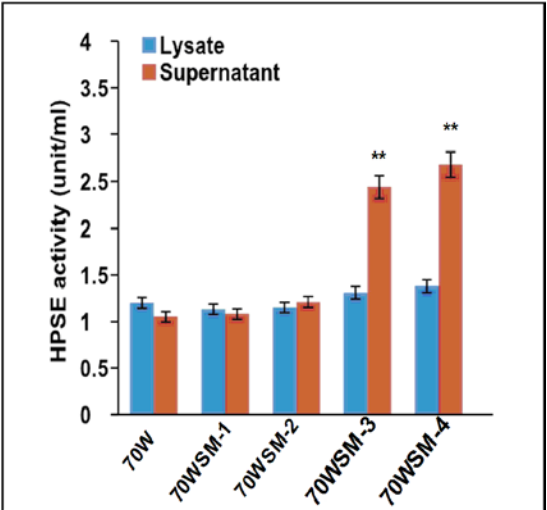
To perform this sub-task, BMM cells were first examined for HPSE expression and activity Higher HPSE expression (**Figure 2**) and activity (**Figure 3**) were detected in 70WSM-3 and 70WSM-4 clones while ELISA assays on these cells showed that secreted HPSE amount in these high BMM cells was approximately 1.2-1.5 fold higher than 70W parental (**Figure 4**).



**Figure 2.** Western blotting analyses of heparanase expression were performed as previously reported (4). Bands corresponding to latent (65 kDa) and active (50 kDa) HPSE could be visualized. Notably, highest levels of active HPSE were detected in 70WSM-3 and 70WSM-4 clones.

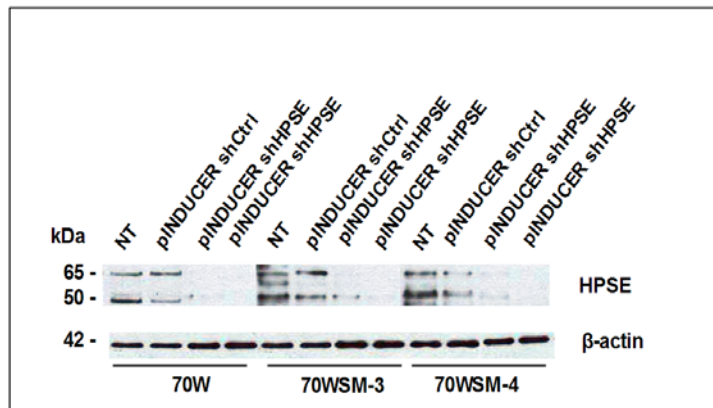


**Figure 3.** Heparanase levels (pg/ml) in cell supernatants and lysates of parental 70W and 70WSM clones measured by ELISA. Highest levels of HPSE could be detected in supernatants of 70WSM-3 and 70WSM-4 clones compared to other 70W selected clones and/or to parental 70W cells.



**Figure 4.** Heparanase activity content of 70W/70WSM clones. HPSE activity was assessed as previously described (4). Highest levels of HPSE activity were detected in supernatants of 70WSM-3 and 70WSM-4 clones respectively, compared to other 70W-selected clones and parental 70W cells.

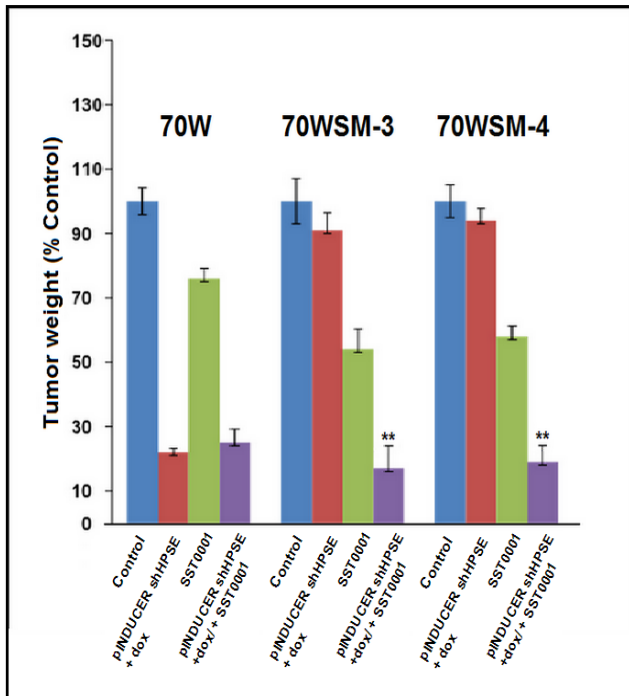
As a second step, to determine whether the pINDUCER lentiviral system yields tractable and inducible RNAi in a cell population, a shRNA targeting HPSE (4) was subcloned into pINDUCER viral vector (pINDUCER shHPSE)(5). Several BMM cells (70W series) were then transduced with pINDUCER10-shHPSE, and transduced cells that survived puromycin selection were cultured in vitro with doxycycline (5), followed by Western blot analysis for HPSE. pINDUCERshHPSE was effective inhibiting the expression of HPSE, either in its latent (65 kDa) or active (50 kDa) forms at multiplicity of infection (m.o.i.) of 5 or above (**Figure 5**).



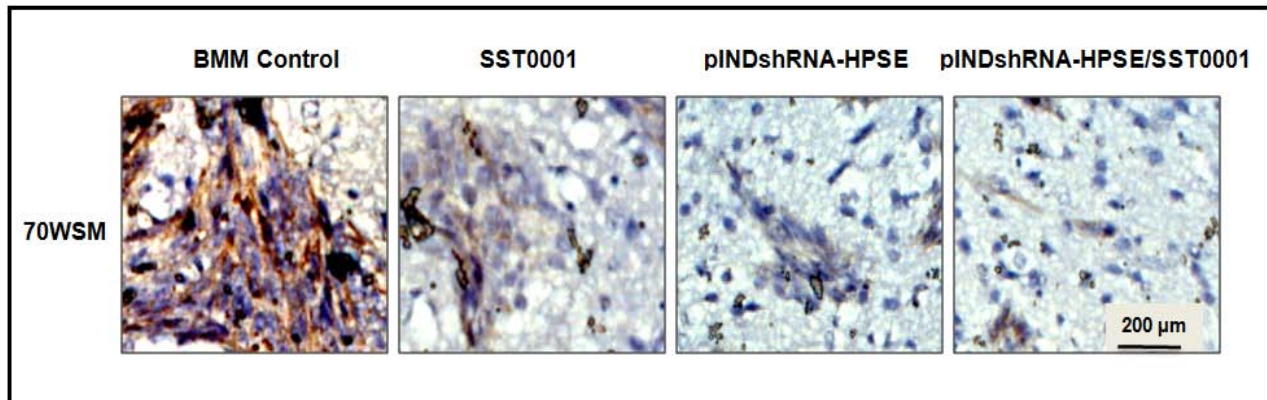
**Figure 5.** Western blotting analyses showing that pINDUCER shHPSE elicits an effective inhibition of latent and active HPSE forms in BMM cells (70W and 70WSM-3/-4, respectively). ShRNA targeting HPSE was subcloned into pINDUCER. BMM cells were then transduced with vectors at two different m.o.i. (5 and 10). Cells were cultured for 4 days with dox, and HPSE levels were then determined. Controls were the use of non-HPSE targeting shRNA and beta-actin levels (loading control).

*Perform in vivo experimental brain metastasis assays in immunocompromised animals injected with BMM cells transduced with pINDUCERshRNA-HPSE.*

To determine whether the pINDUCER shRNA-HPSE could modulate the onset of BMM brain, we tested effects of the inducible HPSE knockdown in BMM cells injected in immuno-compromised mice. We then treated animals with or without SST0001 delivered to animals by using osmotic (Alzet) pumps. Lentiviral-treated BMM cells were injected in mice via intracardiac injection as route of administration. Animals were then administered doxycycline (dox+) or control vehicle (doc) (5), in the absence or presence of SST0001 treatment. The regulation of BMM onset by pINDUCERshRNA-HPSE/SST0001 treatment was then monitored either in the lung or brain of these animals. A synergistic reduction of tumor burden in the lungs (**Figure 6**) and of number of large BMM lesions in these animals was detected (**Figure 7**) in those animals that were treated with SST0001 in combination with pINDUCER shRNAHPSE-transduced cells.



**Figure 6.** SST0001 and pINDUCER shRNA-HPSE synergistically inhibit tumor formation in lungs of treated animals. lungs. pINDUCER-transduced BMM cells were injected intracardiacally in mice (n = 15 per treatment group). Mice were administered doxycycline or vehicle, and treated with or without SST0001 (30 mg/ml dose (30 mg/kg/day for 4 weeks)(7). Tumor growth in lung was then monitored daily by measuring length and width with a digital caliper. Tumors were subsequently harvested and tumor weight calculated and compared among treatment groups. Quantification of tumor formation in lungs of animals treated with or without SST0001/pINDUCER. Results were analyzed for the various conditions tested and were statistically significant (\*\* p<0.01).



**Figure 7.** Immunohistochemical analyses displaying representative BMM inhibition and reduced HPSE expression in BMM of animals injected with high BMM 70WSM cells and following treatment of animals with or without with SST0001 in combination with pINDUCERshRNA-HPSE in the presence of doxycycline (dox+ mice)(5). pINDUCER-transduced 70WSM-4 cells were injected intracardiacally in mice (n = 15/treatment group). Tumor growth in brain was then monitored, animals were sacrificed at set point, brain removed, sections prepared and analyzed for BMM HPSE staining. Shown are representative images of HPSE staining in BMM following the injection of pINDUCER-transduced 70WSM-4 cells for the various conditions tested.

*Implement the use of pINDUCER lentiviral tool kit by cloning HPSE cDNA and perform in vivo experimental brain metastasis assays in immunocompromised mice.*

As a third step, to determine the validity of using HPSE inhibitors in BMM and to implement this sub-task, we inserted human HPSE cDNA (a generous gift from Dr. Motowo Nakajima formerly at Novartis Inc., Takarazuka, Japan) and constructed a pINDUCER cDNA-HPSE (5). We then transduced low-expressing and non-brain metastatic melanoma (BMM) cells, eg, MeWo and SBCI cells (1, 7, 8) with this vector. Animals were then injected with pINDUCER HPSE cDNA-transduced cells. Animals were administered dox (dox+) or vehicle (doc-) in the absence or presence of SST0001 treatment. The regulation of BMM onset by pINDUCER cDNA/SST0001 treatment was subsequently monitored. Mice were sacrificed at set point, brains were removed, and sections were prepared at 300  $\mu\text{m}$  interval through one hemisphere. The presence and the enumeration of BMM large metastasis (50  $\mu\text{m}^2$ ) was then pathologically assessed and determined (**Table 1**).

**Table 1.** In vivo analyses of mice injected with non-BMM (MeWo, SBCI) cells previously transduced with pINDUCERcDNA-HPSE vector and/ treated with or without SST0001.

Cell Line	Treatment	No. mice	Mean No. of BMM
MeWo	pINDUCER cDNA	15	16.6
MeWo	pINDUCER cDNA + SST0001	15	2.9
SBCI	pINDUCER cDNA	15	14.0
SBCI	pINDUCER cDNA + SST0001	15	5.1
MeWo SB1B	Corresponding controls (Vehicle - No treatment)	60	ND



#### **4. KEY RESEARCH ACCOMPLISHMENTS:**

The work reports preclinical findings identifying SST0001 as anti-heparanase compound that inhibits BMM tumor growth in vivo; and coupled with the regulation of BMM with the inducible expression of HPSE at the gene level by performing loss/gain-of function studies.

#### **5. CONCLUSION:**

These results provide mechanistic insights into the anti-HPSE actions of SST0001 and the validity of HPSE as a molecular determinant of BMM onset. This, by having altered either HPSE gene expression (by pINDUCER) or HPSE activity (by SST0001) modulating the onset of BMM in vivo.

#### **6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**

None to report

#### **7. INVENTIONS, PATENTS AND LICENSES:**

None to report.

#### **8. REPORTABLE OUTCOMES:**

None to report.

#### **9. OTHER ACHIEVEMENTS:**

None to report.

#### **10. REFERENCES:**

1. Denkins, Y., Roy, M., Greiter-Wilke, A., Galjour, J., Murry, B., Blust, J., Aucoin, A., Reiland, J., Marchetti, D. Brain metastases in melanoma: roles of neurotrophins. *Neuro-Oncology*, 6(2): 154-165, 2004.
2. Ilan N, Elkin M, Vlodavsky I. Regulation, function, and clinical significance of heparanase in cancer metastasis and angiogenesis. *Int. J. Biochem. Cell. Biol.* 38(12): 2018-2039, 2006.
3. Ridgway L. D., Wetzel M., Marchetti, D. Heparanase effects on proteoglycan – induced signaling in brain metastatic melanoma. *Journ. Cell. Biochemistry*, 111(5): 1299-1309, 2010.
4. Zhang L., Sullivan P.S., Gunaratne P., Goodman J.C., Marchetti, D. MicroRNA-1258

suppresses breast cancer brain metastasis by targeting heparanase. *Cancer Research – Priority Report*, 71(3): 645-6541- 2011. - selected as *Breaking Advances - Highlights from recent cancer literature*. *Cancer Research*, 71 (6): 2025, 2011.

5. Meerbrey K, Hu G, Kessler J, Roarty K, Li M.Z., et al. The pINDUCER lentiviral toolkit for inducible RNA interference *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. (USA)*, 108(9): 3665-3670, 2011.
6. Camacho L, Guerrero P., Marchetti, D. MicroRNA and protein profiling of brain metastasis competent cell-derived exosomes. *PLoS One*, 16; 8(9): e73790, 2013.
7. Ritchie J.P., Ramani V.C., Ren Y, Naggi A., et al. SST0001, a chemically modified heparin, inhibits myeloma growth and angiogenesis via disruption of the heparanase syndecan-1 axis. *Clin. Cancer Res.* 17(6): 1382-1393, 2011.
8. Giovanella B.C., Stehlin J.S., Santamaria C., et al. Human neoplastic and normal cells in tissue culture. Cell lines derived from malignant melanoma and normal melanocytes. *J. Natl. Cancer Institute*, 56: 1131-1142, 1976.

## 11. APPENDICES:

None.

## NOTE:

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